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A PROCEDURE FOR THE REMOVAL OF VESICLES AND PROSTATE SECRETIONS FROM MOTILE RABBIT SPERM CELLS

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Accessory gland secretions and prostatic vesicular bodies were removed from rabbit semen by centrifugation of the sperm cell suspension through a discontinuous percoll density gradient. Purified sperm cells were motile up to 20 hr. after centrifugation although the quality of motility varied between cells obtained from different rabbits. Purified cells were suitable for study of cell motion by the "CELL-SOFT" automated and computerized motion analysis system.

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PREFACE

The work described in this report was authorized under Project Nos. F8J210005 and 1C161102A71A, Research in CW/CB Defense. This work was started in October 1987 and completed in January 1988. The experimental data are contained in laboratory notebook 87-0032.

In conducting the research described in this report, the investigators adhered to National Institute of Health Publication No. 85-23, "Guide for the Care and Use of Laboratory Animals." These investigations were performed in accordance with the requirements of AR 70-18, Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs. In addition, Protocol Number 22-0870340000 was approved by the U.S. Army Chemical Research, Development and Engineering Center, Laboratory Animal Use and Review Committee (LAURC) on 16 July 1987.

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A PROCEDURE FOR THE REMOVAL OF VESICLES AND PROSTATE SECRETIONS FROM MOTILE RABBIT SPERM CELLS

1. INTRODUCTION

Sperm cells collected from rabbits using an artificial vagina are contaminated by fluids from the accessory glands and prostatic vesicular bodies.[1] Accessory gland secretions and prostatic vesicles can be removed by high speed centrifugation of semen through a discontinuous sucrose density gradient.[2,3] Sperm cells so treated are suitable for many biochemical studies, but are immotile. Motile rabbit sperm cells were required to develop an in vitro procedure for screening and assessing the potential cytotoxic and adverse reproductive effects of xenobiotics. This report describes a method for removal of accessory gland secretions and vesicles from rabbit semen without immobilizing sperm cells.

2. MATERIALS AND METHODS

2.1 Animals.

New Zealand white rabbits were individually housed in standard rabbit cages in a room maintained at 75 ± 5 °F and 50 ± 10% relative humidity with a 12-hr light/dark cycle. Standard approved laboratory rabbit chow and water were available ad libitum.

2.2 Collection of Sperm Cells.

Semen was collected using an artificial vagina.[4] Collection frequency was no more than once every three days. A log was kept of the collection frequency and volume of semen obtained from each rabbit.

2.3 Incubation Medium.

The Defined Medium (DM) of Brackett and Oliphant was used to maintain sperm cells.[5] The DM composition is shown in Table 1. A solution of $CaCl_2$ and $MgCl_2$ was prepared separately and added to the solution of the other components. The medium was then exposed to an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 and was simultaneously swirled by hand until the color changed from red to a golden orange. Following pH adjustment, this mixture was sterilized by suction filtration through a 22 µm filter and stored in the refrigerator in a tightly capped culture flask or sterile bottle.

Table 1. Composition of DM

Component	g/L	mM
NaCl	6.550	112.00
KCl	0.300	4.02
$CaCl_2 \cdot 2H_2O$	0.330	2.25
$NaH_2PO_4 \cdot H_2O$	0.113	0.83
$MgCl_2 \cdot 6H_2O$	0.106	0.52
$NaHCO_3$	3.104	37.00
Glucose	2.500	13.90
Sodium pyruvate	0.138	1.25
Bovine serum albumin (fatty acid free, crystalline)	3.000	
Penicillin, sodium salt	0.031	
Phenol red	0.01	
Twice glass distilled water to 1000 mL		

2.4 Percoll Buffer (PB) and Gradient.

The composition of PB is shown in Table 2. Percoll (Sigma Chemical Company, St. Louis, MO) solutions were made by mixing the required amount of percoll, 10x PB, and double distilled water (e.g., for a 50% percoll solution: 5 parts percoll, 1 part 10x PB, and 4 parts water) and warming to 37 °C in a water bath. The discontinuous percoll gradient was prepared by placing the most dense percoll solution in a 15-mL graduated, centrifuge tube and carefully layering successively less dense concentrations of percoll solution using a pasteur pipette.

Table 2. Composition of PB (10x)

Component	g/100 mL	mM
KCl	0.3	4.00
$CaCl_2 \cdot 2H_2O$	0.33	2.25
$MgCl_2 \cdot 6H_2O$	0.106	0.52
NaCl	7.599	130.00
HEPES*	1.192	5.00

The pH is adjusted to 7.4 with NaOH, and volume made up to 100 mL. Before use, 25 mg/mL of glucose and 10 mg/mL of bovine serum albumin are added.

*Sigma Chemical Company

2.5 Centrifugation of Sperm Cells.

Semen from a single rabbit was centrifuged at 400-500 x g for 4-5 min at room temperature (c.a. 23 °C) to remove seminal plasma. The resultant pellet was resuspended in warm (37 °C) DM that had been gassed with a mixture of 5% CO_2, 5% O_2, and 90% N_2 to adjust the pH. The numbers of the sperm cells and particles present were determined by counting an aliquot in a Makler counting chamber (Sefi-medical Instruments, Haifa, Israel) under phase contrast optics (x 100). The sperm cell suspension was layered onto the percoll gradient and centrifuged at 450 x g for 10 min at room temperature.

2.6 Recovery of Sperm Cells.

After centrifugation, the two most dense percoll layers and sperm pellets were combined and diluted with two volumes of warm DM. The suspension was centrifuged at 350-400 x g for 5 min. The resultant pellet was resuspended in 1 mL of DM and incubated at 37 °C under an atmosphere of 5% CO_2, 8% O_2, and 87% N_2 to maintain cell motility. The quality of sperm cell motion was subjectively assessed on a scale of good, fair, or poor. The sperm and particle numbers were measured by counting an aliquot in a Makler Counting Chamber.

2.7 Sperm Swim-Up.

For the swim-up procedure, the sperm pellet, after removal of seminal plasma, was gently covered with 2 mL of warm and gassed DM and placed in an incubator at 37 °C under an atmosphere of 5% CO_2, 8% O_2, and 87% N_2. After 3 hr, approximately 3/4 of the upper portion of the overlaying medium was removed to determine the number of sperm cells that had migrated into the medium and the quality of their motion.

2.8 Sperm Cell Filtration.

A suspension of seminal plasma free sperm cells in warm DM (1-2 mL) was filtered through a column of acid washed glass beads [#5663, R40 (A.H. Thomas, Philadelphia, PA)] prepared in a pasteur pipette. The beads were washed once with 2 mL of DM. The quantity of sperm cells and vesicles in the filtrate and washings was measured by counting an aliquot of the filtrate in a Makler Counting Chamber.

3. RESULTS

3.1 Swim-Up.

Sperm cells from six rabbits were used. Three suspensions of sperm cells were centrifuged in a 12-mL graduated, conical tube to produce a pellet of small surface area. The remaining three suspensions were each centrifuged in a 125- by 15-mm round bottom tube to form a pellet of larger

surface area. Each pellet was gently covered with 2 mL of DM and placed in an incubator at 37 °C under an atmosphere of 5% CO_2, 8% O_2, and 87% N_2. After 1 hr, the bottom 3 mm of medium in one conical tube was cloudy, showing that sperm cells had migrated in significant numbers from the pellet into the medium; the motility grade of sperm cells from this rabbit (#189) was also the highest (Table 3). After 3 hr, the medium overlaying #189 sperm pellet was cloudy with a dense layer above the pellet. The medium in each of the three round bottom tubes was clear, while the bottom 3 mm of medium in each of the remaining conical tubes was cloudy. The medium in all tubes was gently removed using a pasteur pipette fitted with a neoprene bulb. The quantity and quality of the sperm cells were assessed (Table 3). Contamination by vesicles and particulate matter in all sperm cell suspensions was considerable and was not quantified; in additon, recovery of sperm cells was poor (Table 3).

Table 3. Swim-Up Purification of Sperm Cells

		Incubation Time			
		0 Hour		3 Hour	
Rabbit	Tube type	% motile*	Total sperm ($\times 10^7$)	% motile	Total sperm ($\times 10^7$)
189	Conical	89(G)	14.8	88(G)	3.2
567	Conical	81(F/P)	8.4	20(F/P)	0.15
564	Conical	91(F/P)	7.8	23(F/P)	0.13
173	Round bottom	85(F/P)	7.2	24(F/P	0.1
177	Round bottom	70(F/P)	13.2	30(F/P)	0.1
971	Round bottom	80(F/P)	22.4		

*Motility grade: poor (P), fair (F), good (G)

3.2 Filtration Through Glass Beads.

Suspension of rabbit sperm cells in DM was poured onto 2- or 4-cm columns of glass beads prepared in pasteur pipettes, and the beads were washed with DM. The quality and quantity of sperm cells in each eluate and wash were assessed by phase contrast microscopy (Table 4). Recovery of sperm cells was good, but, vesicle contamination in all eluates was heavy, showing filtration through beads was unsuitable as a purification procedure.

Table 4. Glass Bead Filtration of Sperm Cells

Initial Cells			Filtration Conditions			Filtered Cells	
Rabbit	% Motile*	Total Cells	Cell Suspension (mL)	Column Size (cm)	Wash Volume (mL)	% Motile	Total
567	62(P)	8.8×10^7	0.5	2	2 x 0.5	12(P)	$.65 \times 10^7$
177	42(P)	1.3×10^7	0.5	2	2 x 0.5	30(VP)	3.4×10^7
564	90(G)	1.3×10^8	0.5	4	2 x 0.5	85(F/G)	2.7×10^7
562	14(P)	8.8×10^7	0.5	4	2 x 0.5	18(VP)	7.1×10^7
173	91(VG)	2.9×10^8	2.0	4	-	67(F)	1.2×10^8
338	76(G)	6.1×10^7	2.0	4	-	55(F)	9.0×10^7
189	94(G)	2.6×10^8	1.0	2**	2 x 0.5	92(G)	2.5×10^8
337	81(G)	1.3×10^8	1.0	2**	2 x 0.5	92(G)	2.6×10^8

*Motility grade is poor (P), very poor (VP), fair (F), good (G), very good (VG).

**Column washed with DM before filtration.

3.3 Centrifugation Through Percoll Gradients.

Centrifugation of a sperm cell suspension at room temperature (23 ± 5 °C) for either 15 min at 600 x g through a discontinuous 50% (4 mL) and 75% (1 mL) or for 10 min at 500 x g through a 50% (4 mL) and 65% (1 mL) percoll gradient in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 was not as effective in removing contaminating vesicles. The vesicles were found as a thick band on the surface of the 50% percoll solution and in the 50% percoll solution; some were present together with sperm cells in a band at the interface of the two percoll solutions. Sperm cells were found as a pellet, but some were present in the 75% or 65% percoll solution. Sperm counts showed that recovery of sperm cells was >90% with a reduction in vesicle contamination of 85-95%. However, there was a drop in the velocity of cell motion and in the percentage of motile cells. In an effort to maintain the quality of cellular motion, the percoll gradients were prepared in a buffer similar in composition to the DM (Table 2). The sperm pellet obtained after centrifugation (10 min, 500 x g) through a 50% and 65% percoll gradient in this buffer was resuspended in 2 mL of DM and incubated under an atmosphere of 5% CO_2, 8% O_2, and 87% N_2. Sperm counts after 4 hr showed 82% of the cells were motile, but the velocity of motion was poor; the apparent velocity of cells in the 65% percoll solution was much higher than the cells that pelleted.

This result suggests that pelleting compromises cellular activity. A pellet was not obtained when sperm cells were centrifuged for 10 min at 500 x g through a 50% (4 mL), 65% (1 mL), and 90% (1 mL) percoll gradient in PB buffer. Sperm cells were present predominately near the top of the 90% percoll layer, in the 65% percoll layer; and, some together with a small amount of vesicles, were in a thin band at the interface between the 50% and 65% percoll layers. The motility of cells in the 90% and 65% layers was excellent after 4 hr incubation at 37 °C under an atmosphere of 5% CO_2, 8% O_2, and 87% N_2. Increasing the time of centrifugation to 15 or 20 min produced a sperm pellet, whereas few cells moved into the 90% layer after only 5 min centrifugation. Purified sperm cells were recovered from the combined 90% and 65% layers by dilution with 2 volumes of DM and centrifugation at room temperature for 5 min at 350-400 x g. Pellets were obtained when cell suspensions were centrifuged for 5 min at 500 x g, or 10 min at 300 x g. Centrifugation at 250 x g for 5 min did not produce a pellet; the supernatant was cloudy and recovery was poor.

Sperm cells from each rabbit were subjected to the procedure to establish the generality of the procedure and the suitability of the donors for further experiments. The suitability was measured by the degree to which the sperm cells survived the multiple centrifugations. The results are shown in Table 5.

Table 5. Purification of Motile Sperm Cells

	Before Centrifugation			Control*	After Centrifugation**		
Rabbit	% Motile***	Total Cells ($x10^7$)	Total Vesicles ($x10^9$)	% Motile	% Motile**	Total Cells ($x10^7$)	Total Vesicles ($x10^9$)
177	67 (F)	4.8	-	>70 (F/G)	74 (F/G)	2.7	-
337	91 (G)	9.2	2.1	>50 (G)	86 (F/G)	4.3	0.27
562	22 (G)	1.8	-	>50 (G)	19 (P)	1.6	-
339	73 (F)	2.2	-	<50 (F)	51 (F)	1.9	-
341	92 (G)	5.2	1.2	<50 (F)	65 (F)	3.7	0.17
338	96 (VG)	5.2	2.3	<70 (G)	58 (F)	4.3	0.12
336	62 (G)	10.0	-	<5 (P)	57 (P)	10.0	-
340	88 (G)	10.0	6.0	>10 (P)	38 (F/G)	4.2	0.02
943	97 (G)	11.0	2.4	>50 (F/G)	44 (F/G)	10.0	0.008
974	86 (G)	41.0	-	<1 (VP)	18 (P)	14.0	-
189	88 (F/G)	32.0	-	>50 (G)	82 (G)	18.0	0.011
567	88 (F/G)	9.6	5.2	>50 (G)	89 (VG)	40.0	0.1
971	86 (F/G)	24.0	3.9	80 (G)	89 (VG)	-	-
564	90 (G)	56.0	3.2	95 (VG)	56 (G)	-	0.04
173	50 (G)	20.0	0.97	78 (VG)	75 (G)	-	0.01

*Cells not centrifuged but incubated at 37 °C, 5% CO_2, 8% O_2, 87% N_2 in DM. Observed either just before or after centrifuged cells.

**Measurements carried out in cells recovered from 90% and 65% Percoll solution by dilution with 2 volumes of DM, centrifugation at 300 x g for 5 min, resuspension in 2 mL of DM and 4 hr incubation at 37 °C under atmosphere of 5% CO_2, 8% O_2, and 87% N_2.

***Motility grade is poor (P), fair (F), good (G), or very good (VG).

4. DISCUSSION

The success of the swim-up method is critically dependent on the degree of sperm cell activity. Good to excellent motility is required for significant movement of cells into the overlayering medium. Two additional drawbacks of this method are (1) even with cells of good motility a long incubation time is required, and (2) the yield of cells is low. Removal of most of the overlayering medium in order to improve cell recovery invariably disturbs the sperm pellet, resulting in extensive contamination with vesicles. Filtration through glass beads is much faster and cell recovery is better than with the swim-up method. Unfortunately sperm cell quality (i.e., motility) is adversely affected. Prior washing of the glass beads prevents the deterioration of cell motility; but even with this modification, contamination of the filtrate with vesicles is extensive.

The method of choice for removal of vesicles and particulate matter from motile sperm cells is by centrifugation through a discontinuous percoll density gradient. The procedure is rapid, requiring less than 1 hr, and contamination with vesicles is low with a range of 90-99.99% removal of the vesicles. Recovery of cells is good (34-91%), and more importantly, the quality of cell motion (percent motility and velocity of motion) after 4 hr incubation at 37 °C in an atmosphere of 5% CO_2, 8% O_2, 87% N_2 following centrifugation is as good as control cells that had not been centrifuged. Although the quality of motion of centrifuged cells was not as good as control cells after a further 16-18 hr incubation, the vesicle-free cells would be suitable for the automated computerized study of cell motion by the "Cell-Soft" system (Cyro Resources Limited, New York, NY).

5. CONCLUSION

Contaminating vesicles and particulate matter may be removed from rabbit sperm cells by centrifugation through a percoll density gradient. Purified cells remain motile without significant loss of motion quality for a minimum of 4 hr after configuration. These cells are suitable for study of cell motion by the "Cell Soft" automated and computerized system.

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